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14. ABSTRACT Prostate cancer (PCa) remains the most common malignancy and the second leading cause of cancer-related death for men in the United States. Recent studies have shown significant interactions between nerves and adjacent cancer cells that promote cell survival, proliferation and migration of PCa cells. Our studies of laser captured prostate cancer reactive stroma have shown that among the most upregulated genes is glial cell line-derived neurotrophic factor (GDNF), which is expressed by peripheral nerves. GDNF binds to RET, a receptor tyrosine kinase, in conjunction with its co-receptor GFR α 1 (GFR α 1) and activates cellular signaling. Both RET and GFR α 1 are expressed on all PCa cell lines tested and RET protein is increased in PCa. Studies in pancreatic cancer strongly implicate GDNF as a key factor promoting perineural migration. We will test the hypothesis that GDNF is expressed by nerves and that it acts on RET/GFR α 1 in adjacent PCa cells to promote proliferation and invasion and inhibit apoptosis and that disruption of this signaling cascade will inhibit PCa progression <i>in vivo</i> .				
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1. INTRODUCTION.

Prostate cancer (PCa) remains the most common malignancy and the second leading cause of cancer-related death for men in the United States. The tumor microenvironment plays an important role in the initiation and progression of PCa. One important component of this microenvironment is nerves. PCa has a propensity to grow in perineural locations and large diameter perineural tumor is one of the most significant pathological predictors of poor outcome following radical prostatectomy. Perineural invasion is also associated with poor outcomes following radiation therapy. Recent studies have shown significant interactions between nerves and adjacent cancer cells that promote cell survival, proliferation and migration of PCa cells. Recent landmark studies have shown that chemical or surgical ablation of nerves inhibits tumorigenesis and metastasis in both xenograft and transgenic mouse models of PCa, unequivocally establishing that nerve-PCa cell interactions play a significant role in PCa initiation and progression. Interestingly, PCa cells and nerves have bidirectional interactions. Recent studies have shown that PCa induces axonogenesis and neurogenesis and higher nerve densities in the microenvironment are associated with aggressive clinical behavior in PCa. **Thus nerves and PCa cells interact in a potential feed forward loop in which nerves promote prostate cancer cell proliferation, survival and invasion and PCa cells in turn increase the number of nerve fibers via increased axonogenesis and neurogenesis.**

Our studies of laser captured prostate cancer reactive stroma¹ have shown that among the most upregulated genes is glial cell line-derived neurotrophic factor (GDNF), which is expressed by peripheral nerves. GDNF binds to RET, a receptor tyrosine kinase, in conjunction with its co-receptor GFR α 1 and activates cellular signaling. Studies in pancreatic cancer strongly implicate RET signaling as a key factor promoting perineural migration. Our goal is to evaluate the role of RET signaling in the response of PCa to adjacent nerves and to determine if disruption of this signaling cascade will inhibit PCa progression in vivo.

2. KEYWORDS: prostate cancer; nerve, RET, GDNF, GFR α 1

3. OVERALL PROJECT SUMMARY:

Major Task 1. Obtain regulatory approvals for animal and human protocols (Months 1-3)

All regulatory approvals have been obtained and maintained.

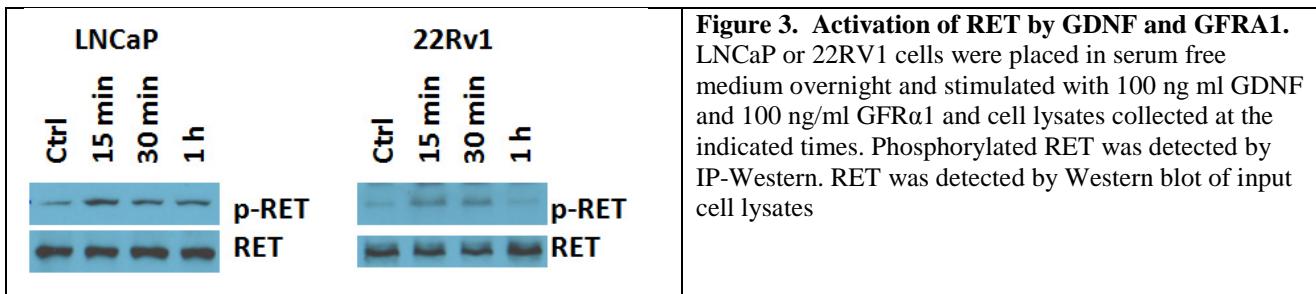
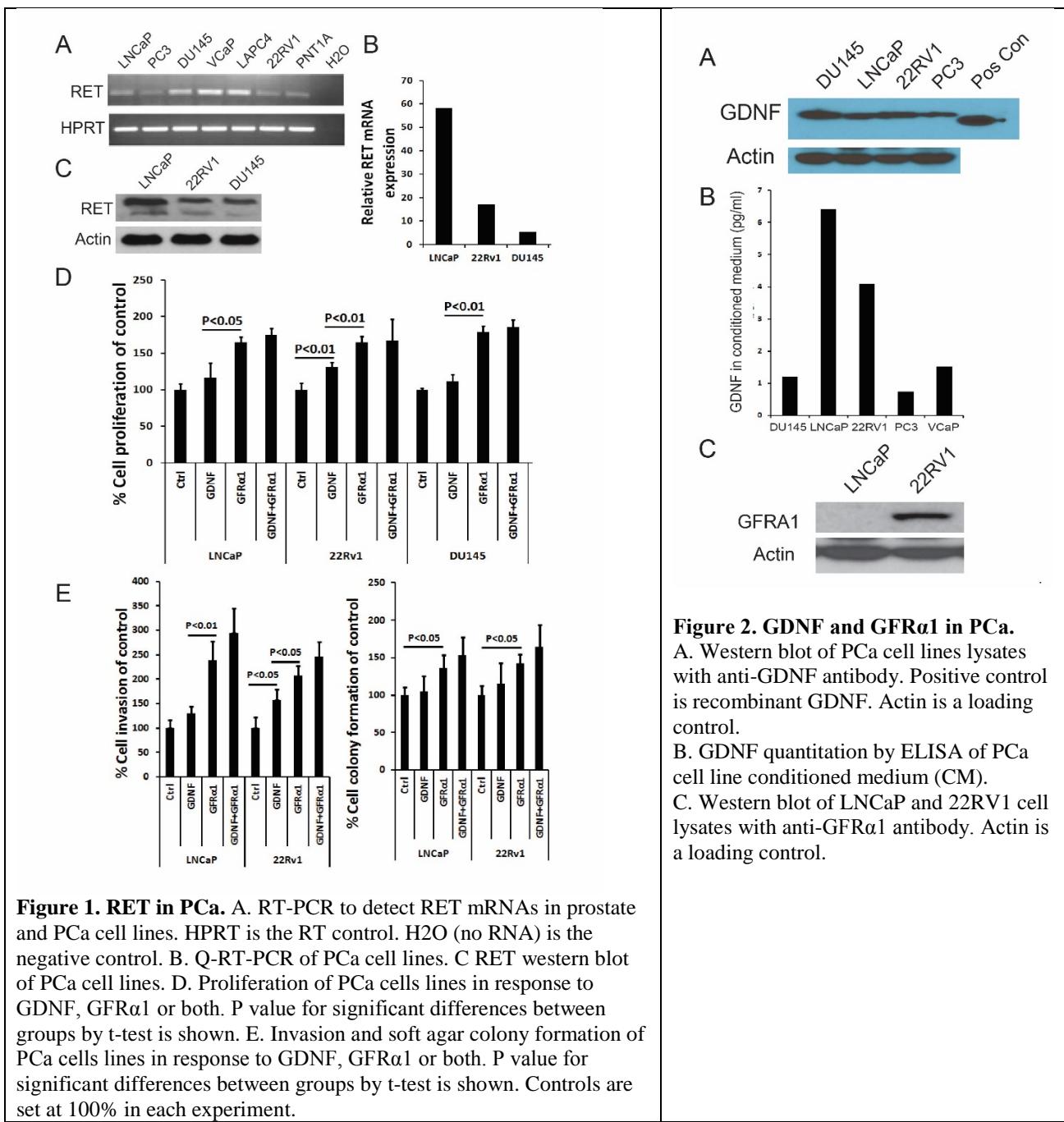
Major Task 2: In vitro studies of GDNF.

Subtask 1: Sub-aim 1.1. Phenotypic effects of GDNF on prostate cancer cells in vitro (months 1-12).

Given that GDNF is secreted in the tumor microenvironment we initial sought to determine if RET is expressed in a broad array of PCa cells. RT-PCR of RNAs from all PCa cell lines and the immortalized prostate epithelial cell line PNT1a shows that all PCa cell lines and prostate epithelial cells express RET mRNA at variable levels (Fig 1A). Western blot analysis confirmed RET expression at the protein level that was concordant with mRNA levels measured by Q-RT-PCR (Fig 1B and 1C). Both GDNF and GFR α 1 are released by nerves and have been previously shown to be able to induce cancer cell proliferation and invasion. We therefore treated PCa cell lines with GDNF, GFR α 1 or both and evaluated proliferation (Fig 1D). Somewhat surprisingly, all cell lines responded to GFR α 1 alone but only 22RV1 cells responded to GDNF alone. Furthermore, combined treatment with GDNF and GFR α 1 was no more potent than GFR α 1 alone. Similar results were seen when invasion and soft agar colony formation were assessed (Fig 1E). These findings implied that GDNF was present in the PCa cell lines since all cell lines responded to exogenous GFR α 1. Furthermore, only 22Rv1 cells can respond to exogenous GDNF, implying they are the only PCa cell line that expresses GFR α 1. Indeed, as seen in Fig 2A, by western blotting and ELISA for GDNF, all PCa cell lines expressed variable levels of GDNF protein. This indicates that GDNF can act as an autocrine factor in PCa, in addition to its role as a potential paracrine factor from nerves. Furthermore, RT-PCR analysis showed that only 22RV1 expressed significant amounts of GFR α 1 and western blot shows that 22RV1 are the only PCa cell lines to express GFR α 1 protein (Fig 2C). It should be noted that even 22RV1 responded to exogenous GFR α 1, indicating that exogenous GFR α 1 protein can enhance the response of PCa cells to GDNF even in the presence endogenous GFR α 1. Interestingly, new data has emerged from the pancreatic cancer literature relevant to our observations. He et al² have recently reported that soluble GFR α 1 is released by nerves and plays a key role in enhancing nerve cancer interactions. We believe that a similar situation applies to PCa.

Subtask 2: Sub-aim 1.2. Pathway activation by GDNF

We have undertaken a series of experiments using western blotting and reverse phase protein arrays to examine pathway activation in PCa cells after activation of RET signaling. As shown in Figure 3, RET signaling is activated with rapid kinetics after stimulation of PCa cell lines with GDNF and GFRA1. RET phosphorylation increased within 15 minutes after stimulation.



In LNCaP cells, GFR α 1 (and GDNF plus GFR α 1) significantly stimulated phosphorylation of ERK1/2, but did not increase AKT phosphorylation, which is already high in these cells due to PTEN inactivation (Fig. 4A). In 22Rv1 cells, GDNF, GFR α 1 and GDNF plus GFR α 1 stimulated AKT phosphorylation, with GFR α 1 and GDNF plus GFR α 1 showed stronger effects (Fig. 4A). ERK1/2 phosphorylation was not increased in 22Rv1 cells which have intrinsically high ERK activation. However, in both cell lines there was a marked increase on phosphorylation of p70S6 kinase. There was also a slight increase in p70S6K protein in 22Rv1 cells (Fig 4A) To dissect the role of AKT and ERK phosphorylation in the activation of p70S6K we treated each cell lines with either a selective PI3K inhibitor, LY294002 or a selective inhibitor of the MAPK pathway, U0126. Both drugs showed the expected activities in both cell lines (Fig 4B). Interestingly, treatment with either LY294002 or U0126 almost completely abolished activation of p70S6K in both cell lines. These results indicate that activities of both PI3K and MAPK pathways are required for activation of p70S6K, although the dominant pathway activated by GDNF/ GFR α 1/RET depends on the cellular context. The induction of p70S6K phosphorylation by RET via these pathways was confirmed by knockdown of RET in both PCa cell lines tested (Fig 5)

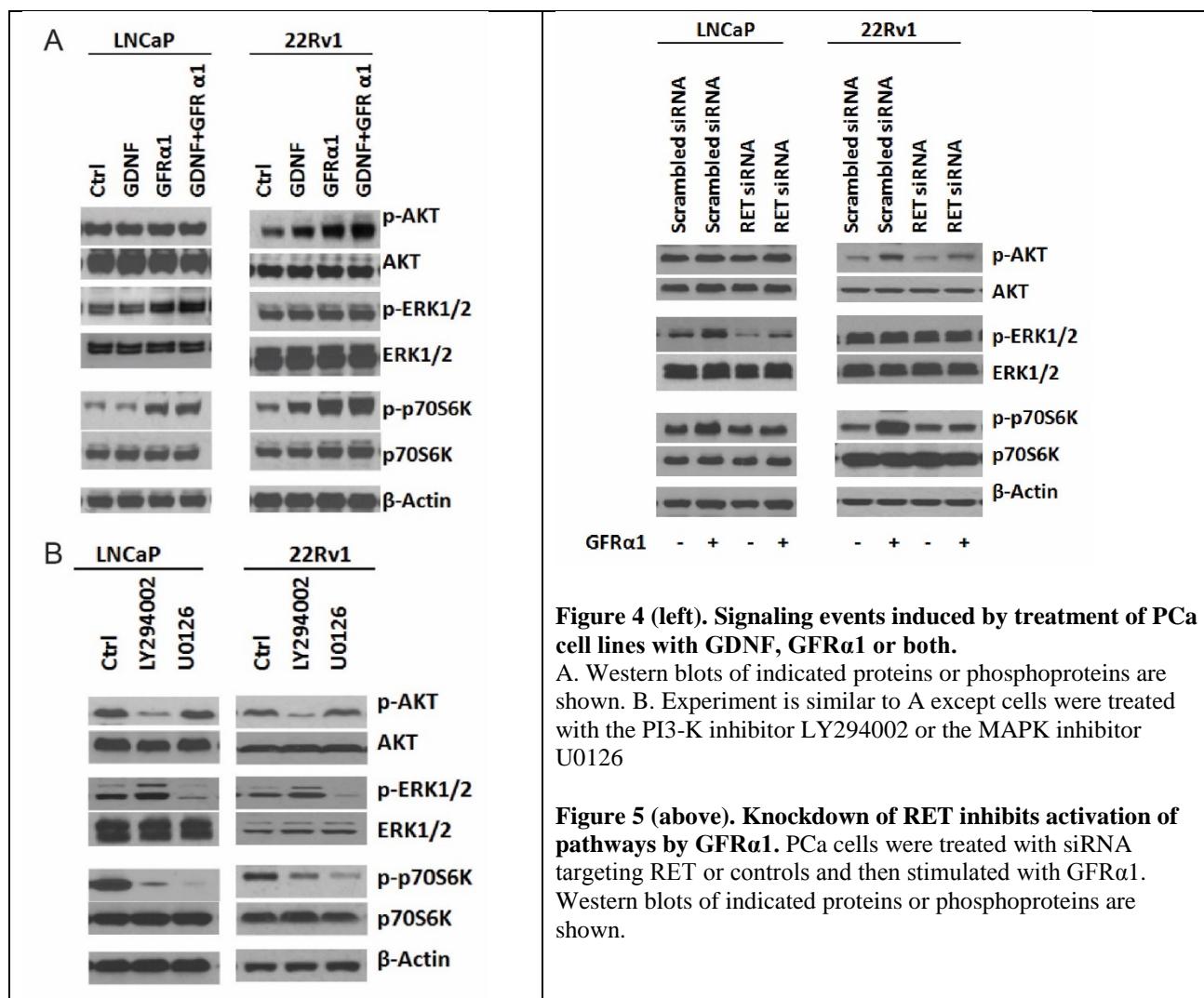
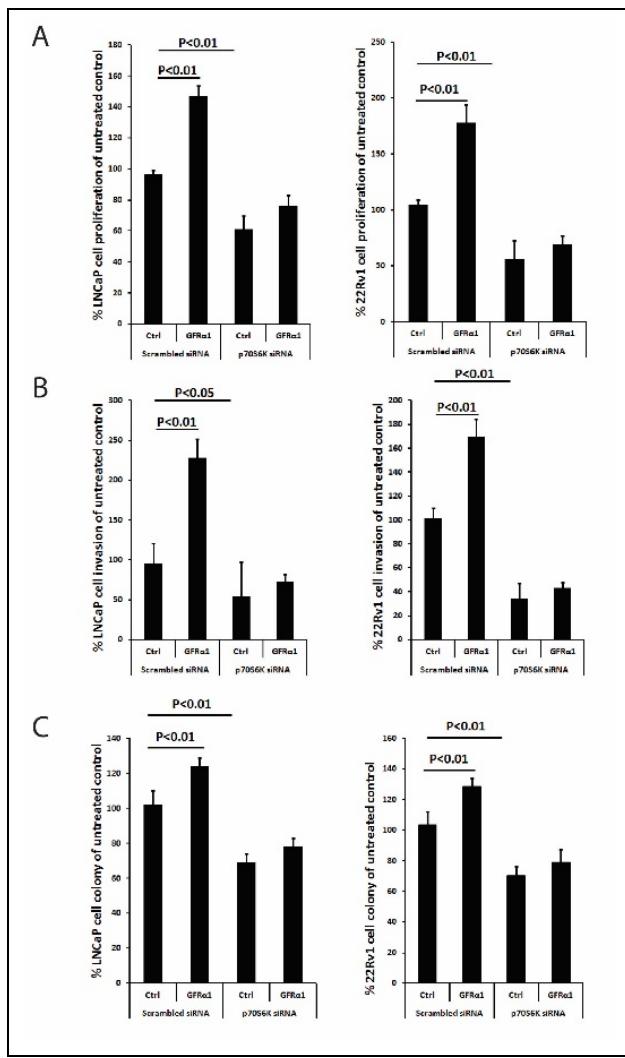


Figure 4 (left). Signaling events induced by treatment of PCa cell lines with GDNF, GFR α 1 or both.

A. Western blots of indicated proteins or phosphoproteins are shown. B. Experiment is similar to A except cells were treated with the PI3-K inhibitor LY294002 or the MAPK inhibitor U0126

Figure 5 (above). Knockdown of RET inhibits activation of pathways by GFR α 1. PCa cells were treated with siRNA targeting RET or controls and then stimulated with GFR α 1. Western blots of indicated proteins or phosphoproteins are shown.



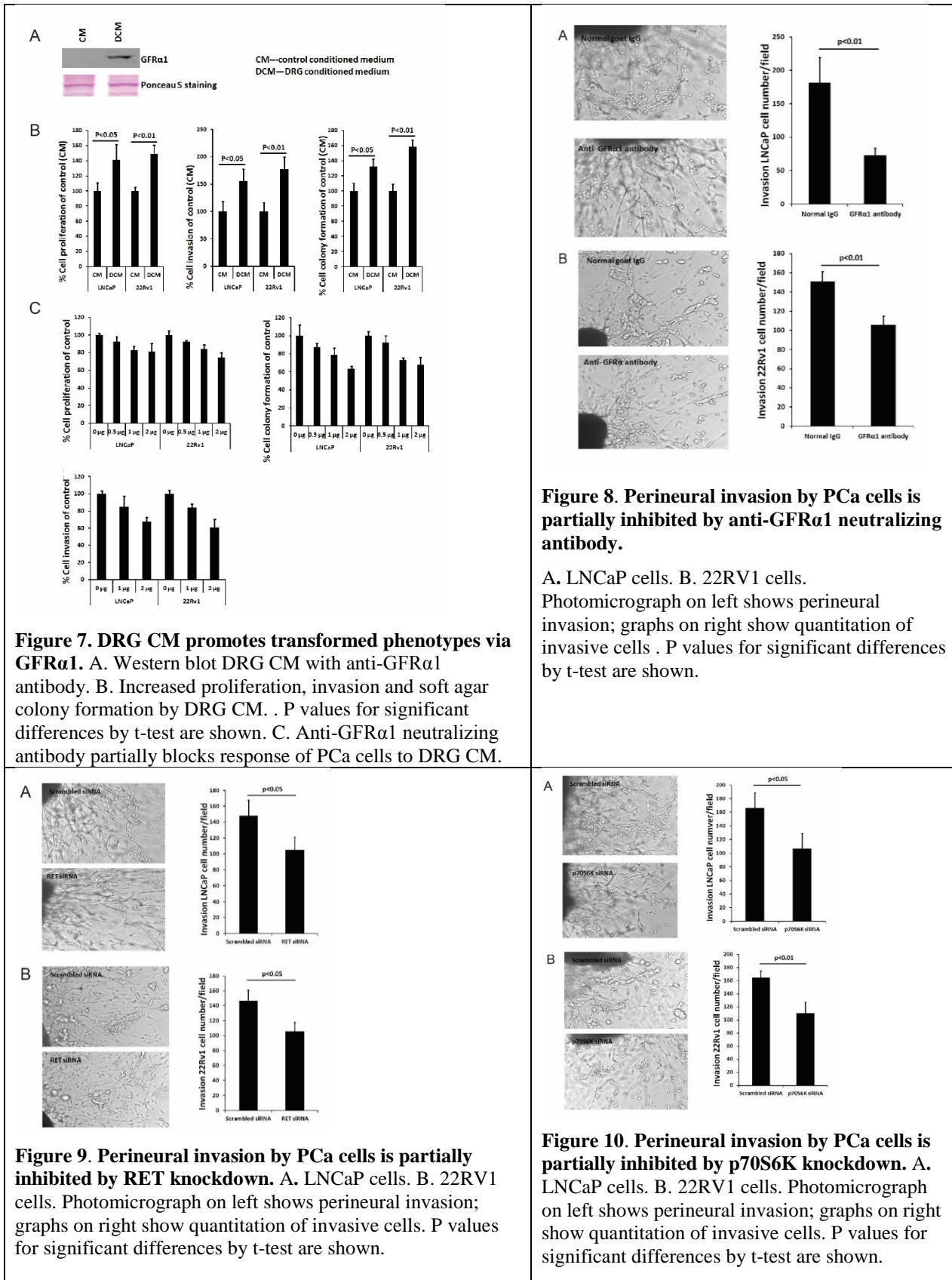
Transient p70S6K siRNA transfections into LNCaP and 22RV1 cells were used to evaluate the role of p70S6K in the effect of GFRα1 on the biological alterations induced by GFRα1. The results show that knockdown of p70S6K markedly reduced cell proliferation (Fig 6A), invasion (Fig 6B) and colony formation (Fig 6C) in response to GFRα1.

Figure 6 (left). Knockdown of p70S6K markedly inhibits GFRα1 induced phenotypes. Cells were pretreated with siRNA targeting p70S6K of control siRNA and treated with GFRα1 and proliferation, invasion or soft agar colony formation measured. P values for significant differences by t-test are shown.

Major Task 3: Role of GDNF in nerve-prostate cancer cell interactions.

It is known that nerves can release soluble GFRα1. We therefore examined the ability of dorsal root ganglia (DRG) excised from mice to enhance cancer phenotypes in PCa cells in vitro. As expected, GFRα1 is present in the conditioned media (CM) of DRG in cultures (Fig 7A). CM from DRG was able to significantly enhance proliferation, invasion and soft agar colony formation in both LNCaP and 22RV1 cells (Fig 7B). This was blocked (although not completely) by neutralizing anti-GFRα1 antibody (Fig 7C).

Using the DRG in vitro coculture model of perineural invasion, we found that LNCaP and 22RV1 cancer cells invaded along the neurites and that perineural invasion was significantly attenuated by anti-GFRα1 antibody (Fig 8), suggesting that GFRα1 also plays a critical role in the perineural invasion. Knockdown of RET with siRNA also markedly inhibited perineural invasion (Fig 9). Moreover, knockdown of p70S6K also decreased perineural invasion (Fig.10). These findings suggest that p70S6K plays a pivotal role in the effect of GFRα1/RET on these cellular behaviors.



Subtask 3: Sub-aim 2.3. Role of RET in prostate cancer progression in vivo (months 4-36)

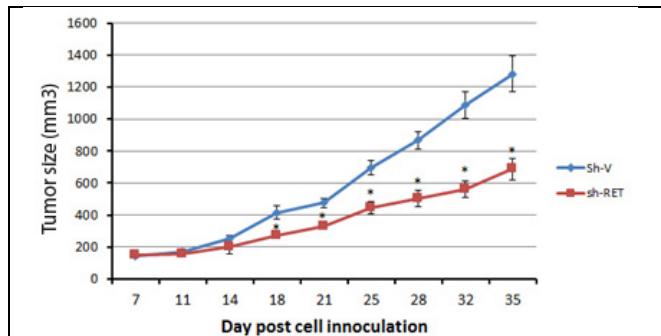


Fig 11. Growth of LNCaP subcutaneous tumors with RET knockdown (red) or vector controls (blue).
Statistically significant differences at each time point are indicated by an asterisk (*).

To determine whether RET signaling promotes tumor growth in vivo we established LNCaP cells with RET knockdown and vector controls. We then evaluated tumor growth in vivo using a subcutaneous xenograft model. As shown in Figures 11 and 12, cells with RET knockdown grew significantly more slowly than vector controls (Fig 11), with lower final tumor weights (Fig 12).

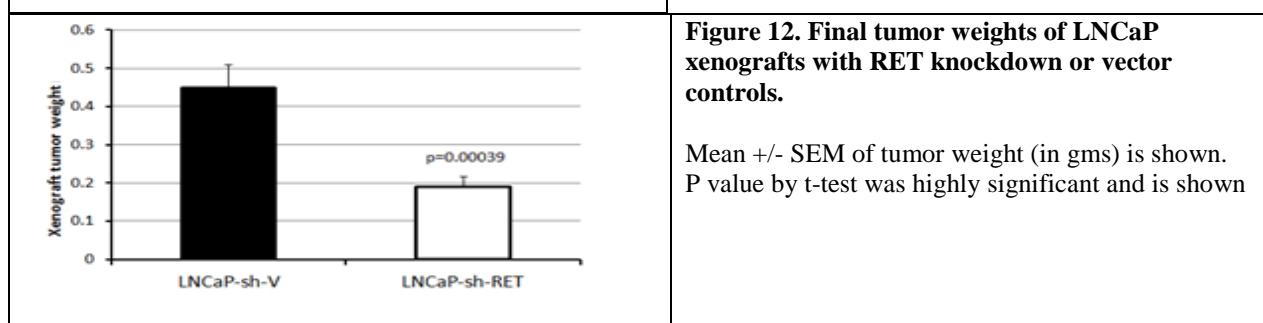


Figure 12. Final tumor weights of LNCaP xenografts with RET knockdown or vector controls.

Mean +/- SEM of tumor weight (in gms) is shown. P value by t-test was highly significant and is shown

Major Task 3: In vivo expression of RET, GFRα1 and GDNF in prostate cancer

We have optimized immunohistochemistry for RET (Fig 13) and stained a large TMA with >500 cases of PCa. This TMA is currently being scored. Given that p70S6K plays a key role in RET signaling in PCa (see above) we have also developed optimized conditions for detecting phosphorylated p70S6K by IHC (Fig 13) and are planning to stain multiple PCa tissues and TMAs in the coming year

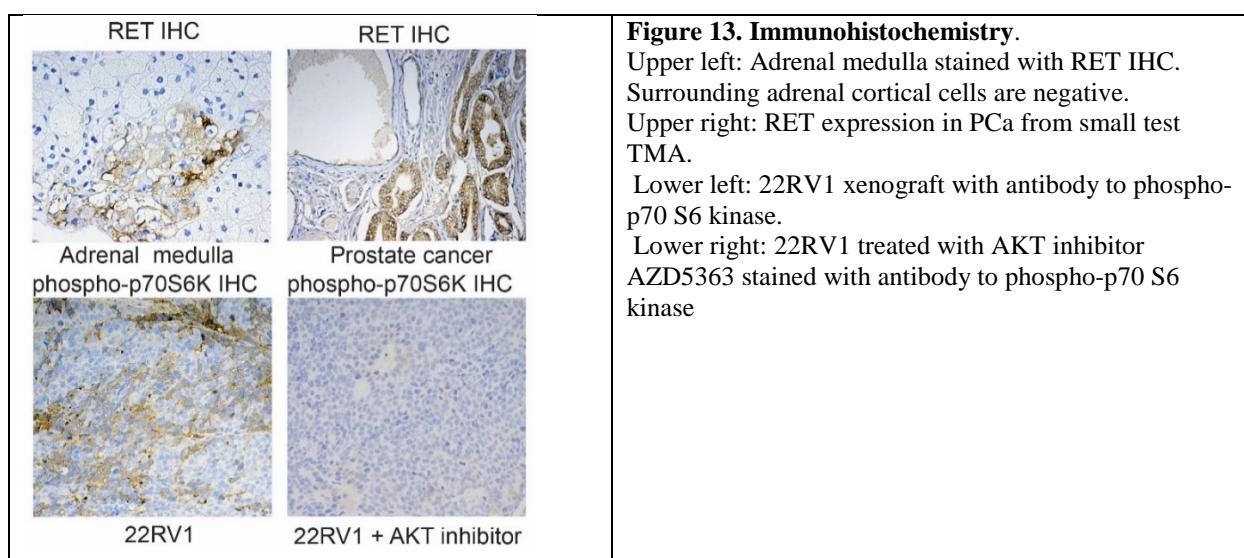


Figure 13. Immunohistochemistry.

Upper left: Adrenal medulla stained with RET IHC. Surrounding adrenal cortical cells are negative.
Upper right: RET expression in PCa from small test TMA.
Lower left: 22RV1 xenograft with antibody to phospho-p70 S6 kinase.
Lower right: 22RV1 treated with AKT inhibitor AZD5363 stained with antibody to phospho-p70 S6 kinase

4. KEY RESEARCH ACCOMPLISHMENTS:

- We have shown that multiple prostate cancer cell lines express RET and respond to RET signaling with increased proliferation, invasion and soft agar colony formation.
- Surprisingly, GDNF is expressed as an autocrine factors by PCa cell lines in addition to be expressed by nerves
- GFR α 1 is only weakly expressed by 22RV1 cells of all PCa cell lines tested and thus RET signaling is dependent on GFR α 1 secreted primarily by nerves to promote RET signaling
- We have shown that GDNF/GFR α 1 activate either ERK and AKT signaling in prostate cancer, which are required to activate the downstream target p70S6 kinase by phosphorylation
- Activation of p70S6K plays a key role in enhancing transformed phenotypes induced by RET signaling
- RET signaling activated by GFR α 1 plays an important role in perineural invasion
- Knockdown of RET significantly inhibits tumor growth in vivo
- We have established optimized immunohistochemistry for RET and phospho-P70S6K and are proceeding with analysis of human PCa tumors

5. CONCLUSION

Our data show that RET is expressed in PCa and responds to GDNF in the presence of GFR α 1 to promote transformed phenotypes including proliferation, invasion, soft agar colony formation and tumor growth in vivo. Somewhat surprisingly, we have found that GDNF is expressed as an autocrine factor in PCa in addition to its paracrine role as a growth factor secreted by nerves. Furthermore, the presence of GFR α 1, which is derived mainly from nerves, appears to be the limiting factor in RET signaling. Thus, a perineural niche favoring RET signaling is created in PCa. RET signaling enhances both ERK and AKT signaling, both of which activate p70S6 kinase. Our data supports the concept that p70S6 kinase is a key downstream target of RET signaling. Our studies indicate that RET signaling is a potential therapeutic target in PCa. RET inhibitors such as lenvatinib (although not specific) are already in clinical use and thus our studies have potential translational implications.

6. PUBLICATIONS, ABSTRACTS, AND PRESENTATIONS: None. We are currently preparing a manuscript to be submitted within the next two months containing data from this progress report.

7. INVENTIONS, PATENTS AND LICENSES: None

8. REPORTABLE OUTCOMES: None

9. OTHER ACHIEVEMENTS: None

10. REFERENCES

1. Dakhova O, Ozen M, Creighton CJ, Li R, Ayala G, Rowley D, Ittmann M. Global gene expression analysis of reactive stroma in prostate cancer. *Clin Cancer Res.* 2009;15(12):3979-89. PMCID: 2734921.
2. He S, Chen CH, Chernichenko N, Bakst RL, Barajas F, Deborde S, Allen PJ, Vakiani E, Yu Z, Wong RJ. GFRalpha1 released by nerves enhances cancer cell perineural invasion through GDNF-RET signaling. *Proceedings of the National Academy of Sciences of the United States of America.* 2014;111(19):E2008-17. PMCID: 4024863.